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The RhoGTPases play a critical role in cell migration via regulation of cytoskeletal changes. Increased expression of Rho proteins, in particular RhoC, have been associated with invasive carcinoma, however, the functional contributions of the individual isoforms have been difficult to evaluate due to insufficient molecular tools. We used a stable retroviral RNAi approach to abrogate expression of RhoA or RhoC in the SUM-159 invasive breast carcinoma cell line. We observed that loss of RhoA stimulates invasion, resulting in enhanced lammellipodia formation and decreased adhesion to laminin-1. Conversely, loss of RhoC diminishes invasive potential and impedes cell spreading and lammellipodia formation. A compensatory relationship between RhoA and RhoC at the level of expression and activation was observed and we postulate the reduction in invasive potential for the RhoC siRNA cells results from loss of RhoC expression, as well as increased RhoA activity. Increased invasion in the RhoA siRNA cells results from decreased RhoA and increased RhoC activity but other factors may also contribute based on the use of C3 transferase to inactivate all Rho isoforms. Preliminary *in vivo* orthotopic studies reveal that RhoC is important for tumour formation while RhoA may regulate the angiogenic response via regulation of VEGF protein expression.

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Introduction

The specific aims of this proposal focus on understanding the contribution of the RhoA and RhoC GTPases to breast carcinoma invasion. Rho GTPases play a critical role in coordinated cell migration via regulation of cytoskeletal changes and are prime candidates for regulating this process in breast cancer, as the transition from a dormant epithelial cell to an invasive cell requires genetic and subsequent cytoskeletal change. Indeed, increased expression of Rho family members, in particular RhoC, have been associated with invasive breast carcinoma, however, the specific functional contributions of the individual isoforms have not been defined. Over the past year, contributions to the study of RhoC have significantly increased, particularly associating expression with tumourigenesis in an expanding number of cell types. Our initial publication from this funding is the first report to show that the RhoA and RhoC isoforms could be distinguished individually by siRNA technology, representing a significant advance over widely used dominant-negative and bacterial transferase approaches. Importantly, using these cell lines in an orthotopic breast carcinoma model is uncovering novel roles for these proteins in the regulation of tumour cell survival and angiogenesis. These data are rapidly advancing our understanding of Rho GTPase and tumour biology.

Body – progress report

Specific Aim 1) Define the relative contribution of RhoA and RhoC to the migration and *in vivo* behaviour of breast carcinoma cells.

Aim 1.1 - Determine the relative expression and activation of RhoA and RhoC

Initial studies focused on a range of breast cell lines, including normal mammary epithelial MCF10A, weakly tumorigenic MCF7, SUM-149 derived from invasive inflammatory breast carcinoma, SUM-159 derived from invasive adenocarcinoma and highly invasive MDA-MB-231 breast carcinoma cells. Relative migratory and invasive potentials were assessed using boyden chamber transwell assays and a range of chemoattractants, including lysophosphatidic acid (LPA), epidermal growth factor (EGF), 3T3 conditioned medium and 10% serum. The most motile cells, MDA-MB-231 and SUM-159, responded indiscriminately to LPA, 3T3 conditioned media and serum, whilst SUM-149 moved only towards 10% serum and MCF7 responded to 3T3 conditioned media. This analysis formed the basis for specific future studies with each cell line.

To correlate Rho protein expression with migration and invasion potential, RT-PCR was performed using primers that distinguish between RhoA and RhoC. No significant differences in expression levels were observed in the panel of cell lines defined above. Western analysis confirmed each line expressed both isoforms, however, the level of expression of either isoform did not correlate with increased invasiveness. Interestingly, the relative expression of RhoA to RhoC varied for each line, and may be more indicative of invasive potential. We chose to pursue our studies primarily using the SUM-159 cell line which was derived from a primary anaplastic breast tumour and is distinct from inflammatory breast cancer and thereby represents an invasive cell line that may be less dependent on RhoC for its motility but more indicative of other highly invasive lines.

This study was restricted due to the availability of reagents as reliable RhoC antibodies are not commercially available. Through our siRNA studies (Aim 1.3) we discovered a polyclonal RhoA antibody (Santa Cruz) also detects the RhoC isoform. Clearly it would be preferable to measure RhoC expression alone, therefore we attempted to generate an antibody with BioSource but after several attempts were unsuccessful. Recently, in collaboration with Bethyl Laboratories, an immunoreactive product has been achieved by depletion of the sera against peptides to RhoA and RhoB and affinity

purification with the immunizing RhoC peptide. Although low titer, it is a positive outcome to move forwards with our analysis.

Aim 1.2 – Determine the impact of increased RhoC expression on migration dynamics

Based on published data that RhoC is over-expressed in tumour cells, and that over-expression in human mammary epithelial cells is associated with increased motility (1, 2) I proposed to investigate its role in motility by over-expression studies. Transient transfection and retroviral infection of SUM-159 cells with wild-type RhoC did not stimulate any significant increase in motility. After defining that RhoC is not expressed exclusively in invasive cells, we decided a loss of function approach would be more suitable.

Aim 1.3 – Dissect the specific contributions of RhoA and RhoC to migration dynamics and in vivo behaviour using RNAi

Rho and RacGTPase activation assays are technically challenging and significant effort was invested in optimising appropriate conditions (including time course on various substratum and a range of activating factors). Using the SUM-159 cells we found both RhoA and RhoC can be activated by brief stimulation with LPA, consistent with published data for RhoA (3), but previously unknown for RhoC. These conditions formed the basis for future analysis.

A stable retroviral siRNA approach was used to knockdown expression of each isoform to investigate their roles in migration and invasion dynamics. Stable lines of SUM-159, MCF7, SUM-149 and MDA-MB-231 cells were generated, however the majority of the work focused on the SUM-159 and MCF7 cell lines as they exhibited the most stable knockdown and reproducible phenotype and are representative of common breast malignancies. The initial characterisation of the RhoA and RhoC siRNA lines was published in Cancer Research in December 2004 (4). The key findings of this manuscript are summarised. Loss of RhoA expression stimulates invasion, resulting in enhanced lammellipodia formation and impaired focal adhesion formation concomitant with decreased adhesion to laminin-1. Conversely, loss of RhoC expression diminishes invasive potential and impedes cell spreading and lammellipodia formation. Interestingly, a compensatory relationship between RhoA and RhoC at the level of expression and activation was observed and we postulate that the reduction in invasive potential for the RhoC siRNA cells results from loss of RhoC expression, as well as increased RhoA activity, consistent with reports in the literature that high RhoA activity is inhibitory to motility (5). Increased invasion in the RhoA siRNA cells results from decreased RhoA expression and low RhoC activity, presuming that low RhoC activity, like low RhoA activity is permissive for increased migration and invasion. The use of C3 transferase to inactivate all Rho isoforms confirmed these hypotheses. In addition, other factors also appeared to contribute to RhoA-associated invasion, with high Rac1 activity observed after LPA stimulation, consistent with published reports on RhoA and Rac1 reciprocity for motility (5). Of note, we did not observe a reciprocal interaction for RhoC and Rac1, suggesting there may be Rac1 independent pathways regulating motility. No alteration in stress fiber formation after LPA stimulation was observed in either cell line and we conclude that such a function must be redundant within the isoforms. The reciprocal compensation between RhoA and RhoC suggests it is not simply the level of expression of one isoform that dictates the invasive potential of a tumour cell line, but rather the relative ratio of the two. Attempts to knockdown both isoforms in the same cell have to date been unsuccessful and we are currently awaiting the production of a dual siRNA retroviral vector.

To assess the function of these genes in a system that more closely resembles the physiological environment of the breast, I designed 3-dimensional (3-D) matrigel assays in which the cells were embedded at low density in matrigel (a reconstituted basement membrane similar to the breast stromal microenvironment). Consistent with the invasion assays, the RhoA siRNA cells formed dense aggregates from which invasive cells emanated, forming a stellate morphology that almost completely filled the well. In comparison, the control cells formed medium sized aggregates with disorganized edges and small stellate projections. The RhoC siRNA cells formed small compact aggregates with limited projections, correlating with their stunted morphology and invasion deficiency. Annexin-V staining of cells dissociated from the matrix revealed no significant difference in the proportion of dead cells, suggesting the RhoC cells are not dying under these culture conditions and are limited in their motility for other reasons. In support of this, rescue of RhoC expression by retroviral infection of the RhoC siRNA cells restored the morphology, resulting in stellate projections similar to the control cells (Figure 1 - Appendix). Whilst no alteration in proliferation was observed in cells growing in monolayer 2-D cultures, it is still possible that proliferation is inhibited in 3-D and therefore contributing to the stunted structures. Attempts to assess cell cycle through FACs analysis failed due to the inability to recover sufficient cells.

To validate the functional effects of these genes *in vivo*, we used a mouse orthotopic model of breast tumourigenesis. Cells ($2x10^6$) were injected in a small bolus of matrigel into the thoracic mammary glands of severe combined immune deficient (SCID) mice. Tumour formation and size was recorded weekly, with termination of the experiment occurring at 10 weeks. Combining the data from two independent experiments, the most striking observation was a lack of tumour formation by the RhoC siRNA cells, with only 3/14 mice forming tumours, which resulted after significant latency, were significantly smaller and apparent histological variants of the control tumours (Dr S Lyle, Pathologist). In contrast, 9/14 mice formed tumours from the control cells and 14/14 formed tumours from the RhoA siRNA cells with no significant difference in the rate of formation. The RhoA siRNA tumours were consistently, although not significantly, smaller than controls which was most apparent during the course of tumour formation rather than at the end of the experiment (end weights; RhoA siRNA, 0.41±0.33g; Control, 0.6±0.35g). No difference in mitotic index (64±9 vs 59±21 respectively) indicates no proliferative defect in the RhoA siRNA cells. Thus, the difference in tumour burden may be attributed to the limited necrosis observed after sectioning (Figure 2 - Appendix), with a more necrotic tumour likely to weigh less than a smaller solid mass. The molecular mechanism for reduced tumour burden and decreased necrosis is the focus of current investigation. A further round of animal experiments is also required to increase numbers for statistical significance.

Angiogenesis, the formation of new blood vessels, is critical to tumour cell survival, growth and metastatic dissemination to distant sites. Vascular endothelial growth factor (VEGF) is a crucial growth factor required for angiogenesis and expression is stimulated by hypoxia during tumour formation. To address the possibility that a reduction in angiogenesis may limit tumour burden in the RhoA siRNA cells, we used an *in vivo* angiogenesis assay. The cells were suspended in matrigel and transplanted subcutaneously in Nude mice on both sides of the flank region (6). After 6 days the mice were sacrificed, images captured of the matrigel plug *in situ*, followed by excision, fixation and staining. Preliminary data from this trial suggest there is more new blood vessel formation in the control cell plugs compared to the RhoA siRNA cells, detectable at both the interface between the smooth muscle wall and penetrating into the cell plug (Figure 3 - Appendix). Quantitation of blood vessel formation by CD31 staining is currently underway along with increasing the number of animals to improve statistical significance.

Complementary to this approach, we investigated VEGF expression in monolayer 2-D and 3-D matrigel outgrowth assays. Under both conditions, cell associated VEGF measured by ELISA (R&D Systems) was significantly decreased in the RhoA siRNA cells (Figure 4 – Appendix). Consistent with our observations, hypoxia-induced RhoA expression can up-regulate HIF-1a expression, leading to the induction of VEGF expression in both renal carcinoma cells (7) and trophoblast cells (8). With the reduction of RhoA expression in the siRNA cells, we could anticipate a reduction in hypoxia-driven VEGF expression and hence reduced angiogenesis. The molecular mechanisms driving this signal cascade are currently unknown. Future studies will aim to evaluate VEGF expression by immunofluorescence in the tumours to confirm the *in vitro* results. We also observed a significant decrease in VEGF expression in the RhoC siRNA cells (Figure 4). This was not surprising as over-expression of RhoC in MCF10A and human mammary epithelial cells has been associated with an increase in secreted VEGF (9, 10). However, no correlation with survival or angiogenesis has been made and our *in vivo* data would suggest RhoC might be important in regulating tumour cell survival during the initiation of tumour formation. Up-regulation of VEGF by breast tumour cells is associated with survival through the formation of autocrine signalling loops (11). These data lead to the hypothesis that Rho proteins impact VEGF signalling and collectively may be responsible for both angiogenic and cell survival responses.

The SUM-159 cells are relatively uncharacterised, but have been reported to be metastatic *in vivo* (12). They form locally aggressive orthotopic tumours, however, on a SCID mouse background, we did not observe any dissemination of cells to predicted lung, liver or bone sites as determined by pan-keratin and vimentin staining. We did however observe the induction of extramedullary hematopoiesis, a condition whereby hematopoiesis occurs outside the bone marrow, typically in the spleen, as the result of recruitment of immune cells such as macrophages and neutrophils to the tumour site (13). The condition occurs with greater frequency and intensity in SCID compared to nude mice and severity is related to the size of the tumour burden (13). In all the control animals we observed significant splenomegaly and myeloid cells in the liver, whilst mice bearing RhoA siRNA tumours were only mildly affected. This could be accounted for by 1) the difference in size of the primary tumour, 2) the reduction in VEGF secretion in the RhoA siRNA tumours and 3) an as yet untested role for colony stimulating factor (CSF) secretion in SUM-159 derived tumours. CSF production in mouse models has been associated with induction of extramedullary hematopoiesis (13) and CSF-1 secretion is clinically associated with aggressive breast tumours by recruitment of macrophages to the site, enhancing metastatic progression (14). Infusion of VEGF at pathological concentrations has also evoked an extramedullary hematopoietic response (15). CSF-1 is also implicated in stimulating an angiogenic response by inducing macrophages to produce VEGF. Our ELISA data indicate that the SUM-159 cells secrete high levels of VEGF. Furthermore, in MDA-MB-231 cells, p115-RhoGEF signalling through RhoA and ROCK has been implicated in up-regulation of macrophage-CSF secretion (16), setting a precedent for Rho regulation of this pathway. The level of CSF-1 secretion in the SUM-159 cell line is currently unknown, but could be a factor stimulating such a physiological response. This awaits future study.

Key Research accomplishments

- Developed a stable retroviral RNA interference strategy for gene knockdown.
- Established siRNA constructs that effectively target RhoA and RhoC isoforms.
- Generated stable RhoA, RhoC and control siRNA lines in SUM-159, MCF7, SUM-149 and MDA-MB-231 cells.
- Demonstrated RhoA and RhoC are both activated by LPA stimulation.
- Demonstrated by the siRNA approach that RhoA and RhoC have distinct functional roles *in vitro*.

  - RhoC expression is pro-invasive
  - RhoA expression impedes invasion
- Developed a 3-D matrigel assay system to recapitulate the *in vivo* microenvironment of the breast. This system also allows complex manipulation of growth conditions by exogenous addition of growth factors and hormones.
- Demonstrated the requirement of RhoC expression for tumour formation and potentially cell survival in the breast microenvironment by regulation of VEGF expression.
- Demonstrated the requirement for RhoA expression for tumour angiogenesis by regulation of VEGF expression.
- Provided further characterisation of the SUM-159 breast carcinoma cell line both *in vitro* and *in vivo*, an important advance in the field given the limited number of invasive breast lines available.

Reportable outcomes

Manuscripts

Simpson KJ, Dugan AS and Mercurio AM. Functional analysis of the contribution of RhoA and RhoC GTPases to invasive breast carcinoma. 2004 Cancer Research 64:8694-8701.

Lipscomb EA, **Simpson KJ**, Lyle S, Ring JE, Dugan AS and Mercurio AM. The α6β4 integrin promotes tumour formation *in vivo* by regulating the expression of vascular endothelial growth factor and tumour survival. In preparation

Abstracts

Oral Presentations

Dana Farber "Breast Cancer Research at Harvard", April 2004. "siRNA defines distinct roles for RhoA and RhoC GTPases in breast carcinoma cells"

Department of Pathology, Postdoctoral Data club series, May 2004. "siRNA defines distinct roles for RhoA and RhoC GTPases in breast carcinoma cells"

Longwood area Mammary gland meeting, September 2004. "Functional analysis of the contribution of RhoA and RhoC GTPases to breast carcinoma invasion"

Lorne Cancer Meeting, February 2005 "Functional analysis of the contribution of RhoA and RhoC GTPases to breast carcinoma invasion"

Poster presentations

Gordon meeting on Mammary Gland Biology, May 2004. "Dissection of RhoA and RhoC specific functions in breast carcinoma cells through stable retroviral RNA interference".

American Society for Biochemistry and Molecular Biology (ASBMB), June 2004. "Dissection of RhoA and RhoC specific functions in breast carcinoma cells through stable retroviral RNA interference".

American Society for Cell Biology (ASCB), December 2004. "Functional analysis of the contribution of RhoA and RhoC GTPases to breast carcinoma invasion".

Harvard Medical School Department of Pathology academic retreat, June 2004. "Dissection of RhoA and RhoC specific functions in breast carcinoma cells through stable retroviral RNA interference".

Other

Over the course of this year I unexpectedly changed laboratories. My original mentor Arthur Mercurio moved Institutes and after much consideration I decided not to join him. In November 2005, I joined the group of Joan Brugge, a well renowned scientist of outstanding merit. Concomitant with this transition, I was promoted to the position of Instructor. The laboratory focus is mammary epithelial cell morphogenesis, survival mechanisms and associated signalling pathways, a highly relevant environment within which to continue my studies. I have just attended the annual retreat for the Department of Cell Biology and found it to be an extremely collegial and collaborative department.

I have been actively involved in the formation of the "Longwood mammary gland meeting", held monthly and targeted to postdoctoral fellows working in basic mammary gland biology within the Harvard Medical area.

I co-chaired the 2003-2004 BIDMC postdoctoral data seminar series, implemented changes to create a more conversive atmosphere within each meeting and enlisted the financial support of several scientific companies to defray the cost of food associated with each meeting.

I initiated orthotopic breast tumour models within the Division of Cancer Biology and Angiogenesis at BIDMC, and trained a number of co-workers for breast and colon models of carcinogenesis and mammary specific clearing of endogenous tissue and fat pad reconstitution studies.

The siRNA constructs generated as part of this proposal were also used by a graduate student in the laboratory to dissect RhoA and RhoC specific functions within a colon carcinoma model for epithelial to mesenchymal transition. Interestingly, we observe a similar functional distinction between the two isoforms, with loss of RhoA expression and up-regulation of RhoC expression requisite for the EMT (Bellovin et al, in preparation). The SUM-159 siRNA lines have also been sent to Dr Stanley Zucker, Veterans Administration Medical Centre New York for investigation of MMP-related invasion and Rho GTPases.

Conclusions

The use of a stable retroviral approach to knockdown expression of highly identical Rho GTPase family members formed the basis of the first year of proposed research. This approach proved to be a highly selective and efficient strategy and we have generated a significant number of resources across a range of breast carcinoma cell lines. The study has highlighted the importance of the contribution of these individual isoforms to carcinoma invasion, both *in vitro* in 2-D and 3-D models and *in vivo*. We have shown RhoA expression impedes invasion and RhoC expression stimulates invasion, however, there is also some level of cross-talk between these two isoforms. We have confirmed published reports that RhoA and Rac1 GTPases share signalling cross-talk for acquisition of motility and advanced the field by showing that RhoC and Rac1 do not. Of the most direct relevance to breast carcinoma invasion, we have investigated the role of these proteins *in vivo* using an orthotopic mouse model. We have demonstrated a previously undefined role for RhoC in the initiation of tumour formation and suggest that this may occur through regulation of VEGF signalling. Given that RhoA expression is also elevated in primary tumours (17), it is likely to contribute to tumourigenesis in other ways in addition to invasion. The *in vivo* studies have shown that RhoA expression is not required for tumour formation, but rather appears to impact angiogenesis via up-regulation of VEGF expression.

Immediate future directions aim to consolidate the link between the Rho GTPases and tumour cell survival and angiogenesis. There is little known of the signalling pathways linking Rho regulation of VEGF. This area is crucial to the formation of tumours and metastatic dissemination to secondary sites. These data also suggest that differential targeting of these isoforms may be feasible for therapeutic purposes, especially in light of the fact that RNAi is currently being developed for clinical therapies.

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Appendices

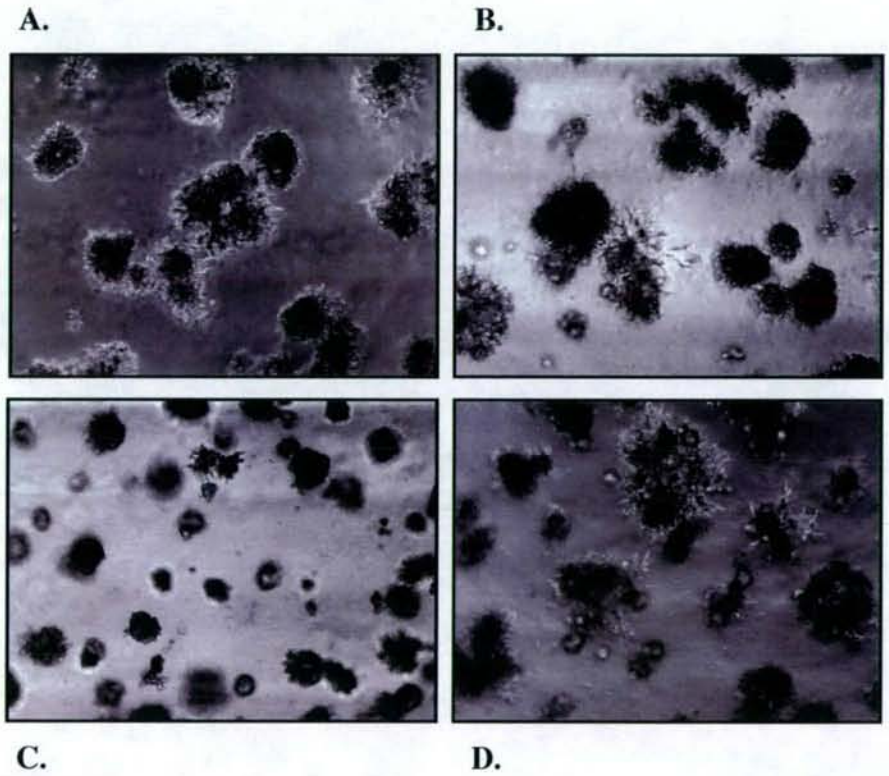

Figure 1. Re-expression of wild-type RhoC in RhoC siRNA cells rescues the stunted morphology in 3-D matrigel assays. Control siRNA expressing empty vector (A) and wild-type RhoC (B) show no significant difference in the stellate morphology indicative of motile cells. RhoC siRNA cells show stunted morphology (C) which is rescued and comparable to control cells after re-expression of RhoC (D). Cells were cultured in matrigel for 11 days, photographed under bright field optics at 4X magnification.

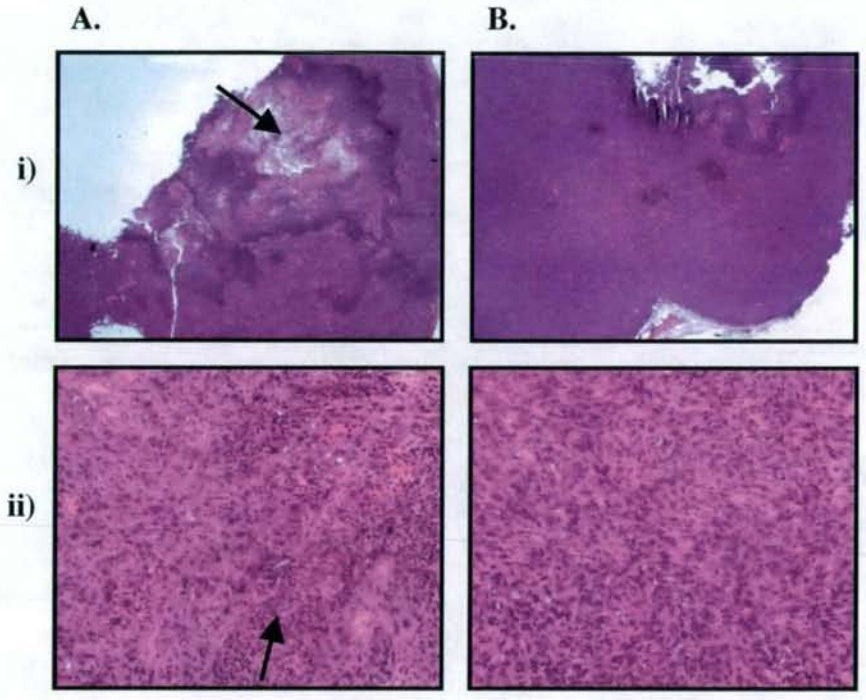

Figure 2. Comparison of representative tumours from control (A) and RhoA siRNA (B) cell lines. The necrotic central portion of the control tumour is indicated by an arrow (Ai) and is relatively absent in the RhoA siRNA tumour (Bi). Higher magnification images (20X) highlight the level of infiltrating host cells in the control (arrow, Aii) relative to the RhoA siRNA tumour (Bii).

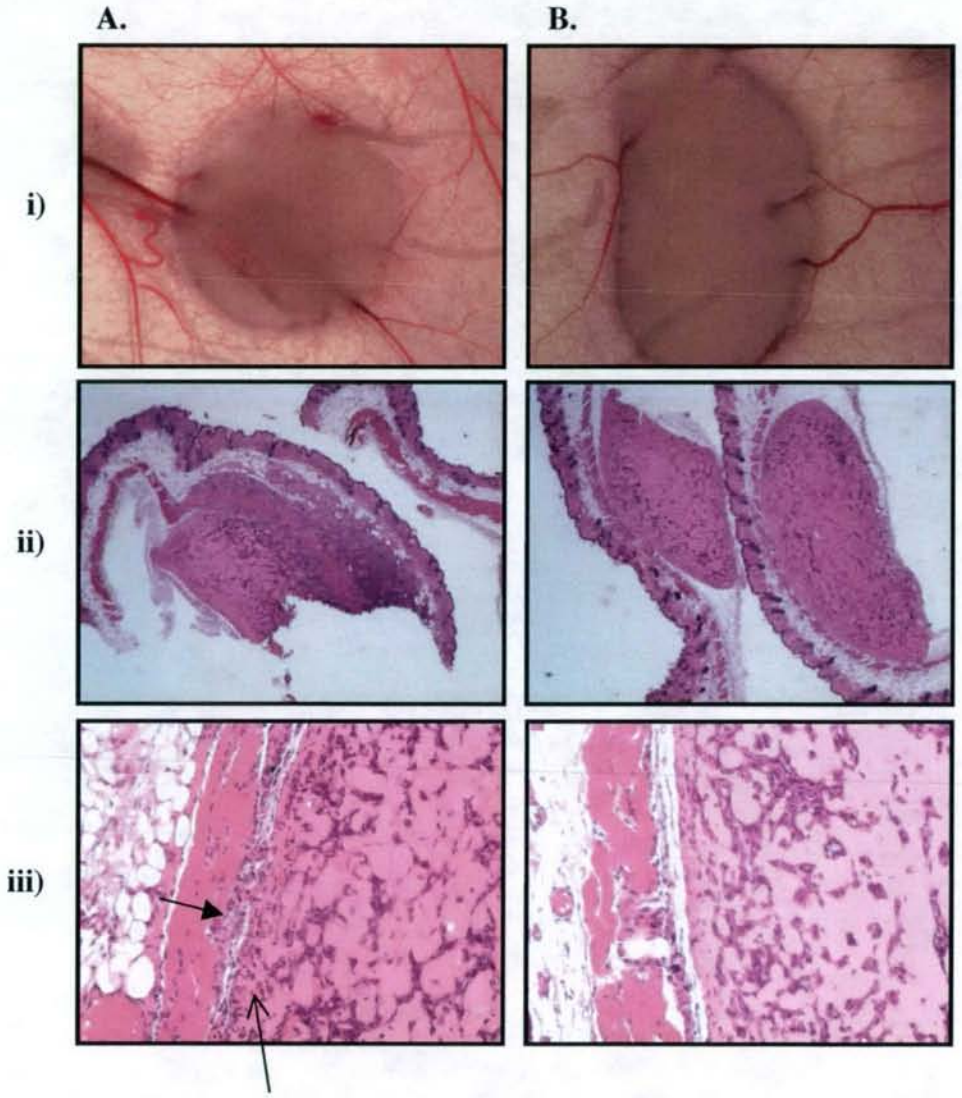

Figure 3. RhoA is required for angiogenesis at the tumour site. Angiogenesis plug assays reveal increased new blood vessel formation in response to matrigel plugs containing control cells (A) compared to the RhoA siRNA cells (B). H&E stained sections at i) 2X and ii) 20X magnification show increased penetration of the smooth muscle layer (solid arrow) by the Control cells and new blood vessel formation (open arrow). New blood vessel formation is markedly absent in the RhoA siRNA plug (Bi and ii).

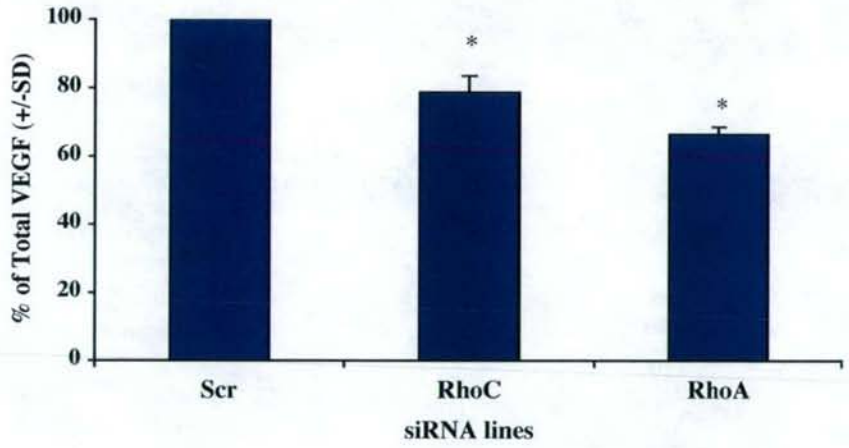

Figure 4. Rho GTPase expression is associated with VEGF induction in 2-D proliferating cells. VEGF was measured by ELISA from cells cultured to 80% confluence. A significant decrease in the proportion of total VEGF was observed after knockdown of RhoC and RhoA expression. Data represents N=3 ± SD. T-test, *$p<0.05$. A similar result was obtained with cells cultured in 3-D matrigel assays.

[CANCER RESEARCH 64, 8694–8701, December 1, 2004]

Functional Analysis of the Contribution of RhoA and RhoC GTPases to Invasive Breast Carcinoma

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ABSTRACT

Although the RhoA and RhoC proteins comprise an important subset of the Rho GTPase family that have been implicated in invasive breast carcinomas, attributing specific functions to these individual members has been difficult. We have used a stable retroviral RNA interference approach to generate invasive breast carcinoma cells (SUM-159 cells) that lack either RhoA or RhoC expression. Analysis of these cells enabled us to deduce that RhoA impedes and RhoC stimulates invasion. Unexpectedly, this analysis also revealed a compensatory relationship between RhoA and RhoC at the level of both their expression and activation, and a reciprocal relationship between RhoA and Rac1 activation.

INTRODUCTION

A critical step in cancer progression is the ability of tumor cells to invade surrounding tissues, a process requiring altered adhesion and polarization, cytoskeletal rearrangement, and basement membrane remodeling (1). Subsequent to invasion, tumors metastasize to distant organs, resulting in morbidity and mortality. Identifying the mechanisms by which cells acquire such invasive potential is crucial for developing strategies to impede carcinoma progression in a clinical setting (2). Analysis of tumor-derived cell lines and primary tumors from breast and other cancers has identified a number of candidate molecules that regulate cell migration and invasion as potential targets for intervention, especially members of the Rho GTPase family (reviewed in ref. 1). This family includes >20 proteins, of which the prototypic members RhoA, Rac1, and Cdc42 have been best characterized. These proteins regulate many cellular functions that underlie cancer progression including cell cycle, gene expression, focal adhesion assembly/disassembly, and matrix remodelling, and they are activated by signals transduced through tyrosine kinase receptors, G protein-coupled receptors and integrins (reviewed in ref. 3).

The Rho-related members, RhoA, RhoB, and RhoC share high sequence identity. These GTPases have been implicated in the progression of tumors from a broad range of cellular origins, and analyses at both the RNA and protein level have correlated their increased expression with tumor progression (4–6). Early studies in this field noted the transforming ability of active RhoA in 3T3 fibroblasts (7). More recently, RhoC has attracted substantial interest with its increased expression being linked to increased invasion in breast (8), melanoma (9), pancreatic (10), colon (4), bladder (11), hepatocellular (12), non–small-cell lung carcinoma (13), and gastric (14) primary tumors or cell lines. Indeed, most studies in this area are based on the assumption that increased Rho protein expression is indicative of enhanced function. Given that the activity of Rho proteins dictates their functionality, the existing data from primary tumors, as well as from many cell lines, are limiting and cannot address this issue specifically.

Analysis of the contribution of the RhoA and RhoC isoforms to aggressive disease has been hampered by a lack of appropriate molecular tools. Seminal studies on Rho protein function have used dominant-negative and constitutive-active approaches, alongside biochemical ablation by *Clostridium difficile* toxin or *Clostridium botulinum* C3 exoenzyme treatment (reviewed in ref. 15). These methods however, are unable to distinguish among individual Rho isoforms because of their high sequence similarity, particularly at regions of functional importance. Recently, Wang *et al.* (16) showed that the Rho proteins are functionally distinct through the use of chimeric p190RhoGAP proteins specific for each isoform. However, this approach still relies on exogenously coexpressed proteins in model cell lines.

To advance our understanding of Rho protein biology, it has become increasingly clear that the functionality of the individual isoforms needs to be determined. To dissect the specific contributions of RhoA and RhoC to the behavior of invasive breast carcinoma cells, we used the SUM-159 cell line. This cell line model, which expresses both Rho proteins, was derived from a primary anaplastic breast tumor and has been shown to be highly invasive in both *in vitro* assays and in *in vivo* orthotopic models of mouse mammary tumorigenesis (17). We have used a stable retroviral small interfering RNA (siRNA) approach to selectively knock-down expression of the *RhoA* and *RhoC* isoforms. Using these cell lines, we were able to define distinct functions for these Rho isoforms and to uncover unexpected relationships between them.

MATERIALS AND METHODS

Antibodies and Reagents. The suppliers, catalogue number and working dilutions of all antibodies used are as follows: Anti-RhoA polyclonal (Sc-179, 1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA); anti-Rac1 monoclonal (RS6220, 1:500, Transduction Laboratories, Newington, NH); antirabbit actin (A0266, 1:2,000, Sigma, St. Louis, MO), phalloidin-FITC (P-5282, 200 mmol/L, Sigma), antirabbit-horseradish peroxidase (711-035-152, 1:10,000, Jackson ImmunoResearch, West Grove, PA), antimouse-horseradish peroxidase (715-035-151, 1:10,000, Jackson ImmunoResearch). Palmitoyl-lysophosphatidic acid was purchased from Avanti Polar Lipids (857-123, Avanti Polar Lipids, Alabaster AL).

Generation of siRNA Constructs. Nucleotide sequences of 19 bp specific to RhoA and RhoC were generated by Oligoengine[1] and BLAST[2] to determine specificity. The RhoA-specific sequence was scrambled to generate a negative control, and confirmation of its nonspecificity was established by BLAST. The target sequences formed part of a larger 64 bp cassette when inserted in both the sense and antisense orientation within the context of a stem loop sequence structure as per Oligoengine (Seattle, WA) design specifications. The 64 bp oligonucleotides were synthesized in the forward and reverse orientation, annealed, and ligated into the pSUPER vector backbone (18). The presence of the insert was determined by sequencing. After confirmation of target sequence specificity by exogenous overexpression approaches (data not shown), the cassette was excised by *EcoR*I and *Xho*I double digestion and subcloned into the prepared pSUPER.Retro backbone (19).

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[1] www.oligoengine.com.

[2] www.ncbi.nlm.nih.gov/blast.

Cell Lines. The SUM-159 and SUM-149 cell lines were obtained from Dr. Steve Ethier, University of Michigan (17), MCF-7 and MDA-MB-231 cells were obtained from the Lombardi Cancer Center (Georgetown University), and MCF-10A cells were obtained from Dr. Joan Brugge (Harvard Medical School). SUM-159 and SUM-149 cells were cultured in HamsF12 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% fetal bovine serum, insulin (5 μg/mL I5500, Sigma), hydrocortisone (1 μg/mL H4001, Sigma), penicillin (100 units/mL), and streptomycin (100 μg/mL, both from Life Technologies, Inc.). MCF-7, MDA-MB-231, and 293T cells were cultured in low glucose DMEM supplemented with 10% fetal bovine serum and antibiotics. MCF-10A cells were cultured as described previously (20). 293T cells were transfected at 50% confluence by a Lipofectamine (Life Technologies, Inc.) complex containing a ratio of envelope plasmid (1.75 μg), packaging plasmid (3.25 μg) and pSUPER.Retro expressing the RhoA, RhoC, and Scr sequence siRNAs (5 μg) in Optimem (Life Technologies, Inc.). Two days after transfection, the virus was harvested, clarified, and the supernatent filtered through a 0.22-μm filter to be used immediately or stored at −80°C. Recipient SUM-159 or MCF-7 cells were plated to reach 50% confluency after 24 hours, and virus was added to the cells at a ratio of virus:fresh media containing Polybrene (8 μg/mL) of 1:1 and 1:8. Puromycin selection (4 μg/mL) in standard growth media was added 24 hours after infection and reduced to 2 μg/mL after 4 days.

Reverse Transcription-PCR. Oligonucleotides were designed to human RhoA, RhoC, and glyceraldehyde-3-phosphate dehydrogenase by the Primer cgi database.[3] RhoA forward was 5′-ATGGCTGCCATCCGGAAGAAA-3′ and reverse 5′-TCACAAGACAAGGCAACCAGA-3′; RhoC, forward was 5′-ATGCGTGCAATCCGAAAGAAG-3′ and reverse 5′-TCAGAGAATGGGACAGCCCCT-3′; and glyceraldehyde-3-phosphate dehydrogenase, forward was 5′CCTGGCCAAGGTCATCCATGAC-3′ and reverse 5′ TGTCATACCAGGAAATGAGCTTG-3′. Total RNA was extracted from adherent cells using RNeasy columns (Qiagen, Valencia, CA). Reverse transcription semiquantitative PCR was performed using 1 μg of total RNA in the One-step RT-PCR reaction kit (Qiagen) as follows; 15 and 20 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 60°C for 60 seconds.

Invasion and Migration Assays. For invasion assays, the upper surface of transwell chambers (8-μm pore, Costar, Cambridge, MA) were precoated overnight with 0.5 μg Matrigel (Becton Dickinson, Franklin Lakes, NJ) diluted in PBS. For migration assays, both the upper and lower surfaces of the transwell chamber were coated overnight with Vitrogen collagen (15 μg/mL; Cohesion, Palo Alto, CA) diluted in PBS. Cells were harvested at 70 to 80% confluence by trypsinization and washed three times in low glucose DMEM containing heat inactivated fatty acid-free BSA (0.25%, DMEM/BSA). The coated surfaces of the transwells were blocked with DMEM/BSA for 30 minutes at 37°C. Cells (1×10^5 or 3×10^5 in a total volume of 100 μL) were loaded into the upper chamber and lysophosphatidic acid (LPA, 100 nmol/L in DMEM/BSA) or NIH-3T3 conditioned media was present in the lower chamber. Invasion assays proceeded for 2 hours, whereas migration assays went for 24 hours at 37°C. For both assays, the upper chamber was swabbed to remove residual cells and fixed with methanol. Invasion assays were stained with crystal violet (0.2% crystal violet in 2% ethanol), rinsed in water, and air-dried. Migration assays were mounted in 4′,6-diamidino-2-phenylindole mounting media (Vector Laboratories). The number of cells penetrating the membrane were determined for five independent fields in triplicate with a 10× objective and bright-field optics or fluorescence and quantitated with IPLab spectrum software (Scanalytics, Fairfax, VA).

Morphological Analysis. Cells were harvested as described. Glass coverslips were coated with laminin-1 (20 μg/mL). The substratum was blocked with DMEM/BSA, and cells (1×10^5) were plated and incubated at 37°C for 60 minutes then treated with or without LPA (100 nmol/L in DMEM/BSA) for 3 minutes. Cells were fixed [2% paraformaldehyde, 200 mmol/L KCl, 20 mmol/L Pipes (pH 6.8), 14% sucrose, 4 mmol/L $MgCl_2$ EGTA; ref. 21], permeabilized in the presence of 0.2% Triton-X-100, blocked in 0.1% BSA in PBS, and stained with FITC-conjugated phalloidin. After washing three times with PBS, cells were mounted, and images were captured under oil immersion at 60× magnification by IPLab spectrum imaging software and a Nikon Diaphot 300 microscope. Quantitation of the lamellipodial area of LPA-treated cells was determined by outlining the area of the lamellae for at least 20 individual cells and quantitating the total surface area encompassed by the lamellipodia with IPLab spectrum software.

Adhesion Assays. Forty-eight well tissue culture dishes were coated with laminin-1 (20 μg/mL, generously supplied by Dr. Hynda Kleinman, National Institute of Dental Research, Bethesda Maryland) or BSA (20 μg/mL) diluted in PBS. Cells were harvested as described and 1×10^5 cells plated per well for 60 minutes at 37°C. After incubation, cells were washed twice with PBS, fixed with methanol, stained with crystal violet (0.2% crystal violet in 2% ethanol), and washed twice with water. Plates were air-dried briefly, and the crystal violet was solubilized by the addition of 200 μL of 1% SDS. The absorbance of 100 μL was read at 595 nm in a microtiter plate reader. Nonspecific adhesion to BSA was used as a baseline to subtract from laminin-1–specific adhesion.

Immunoblotting. Whole cell lysates were prepared by lysis in ice-cold RIPA buffer [50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 10 mmol/L EDTA, 1% NP40, 1% deoxycholate, 0.1% SDS, 2 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 5 μg/mL aprotinin, leupeptin, and pepstatin]. Lysates (35 μg) were separated by electrophoresis through 12 or 15% SDS-PAGE and transferred to Hybond enhanced chemiluminescence membrane (Amersham Biosciences, Piscataway, NJ). Membranes were blocked, hybridized overnight with anti-Rho or anti-Rac1 antibodies (Abs) followed by secondary peroxidase-conjugated antirabbit or antimouse Abs, and detection was by chemiluminescence.

Rho and Rac1 Activity Assays. Rho and Rac activity assays were done following published protocols (22, 23). The glutathione *S*-transferase (GST)-Rho binding domain (RBD) (for Rho activity) and GST-Pak binding domain (PBD) (for Rac activity) fusion proteins were extracted and used to coat glutathione Sepharose (GST) beads. Cells were harvested as described and between 2.5 and 3×10^6 cells were attached to laminin-1 (20 μg/mL diluted in PBS) coated 60-mm dishes for 60 minutes followed by treatment with or without LPA (100 nmol/L) for 3 minutes. Cells were lysed by addition of ice-cold lysis buffer [Rho, 50 mmol/L Tris (pH 7.2), 500 mmol/L NaCl, 10 mmol/L $MgCl_2$, 1% Triton-X-100, 0.5% deoxycholate, and 0.08% SDS; Rac, 50 mmol/L tris (pH 7.4), 100 mmol/L NaCl, 1% NP40, 10% glycerol, 2 mmol/L $MgCl_2$, both containing 2 mmol/L phenylmethylsulfonyl fluoride, and 5 μg/mL each of aprotinin, leupeptin, and pepstatin]. Lysates were clarified and an aliquot (0.5 total volume for Rho and 0.1 total volume for Rac) removed to represent the total lysate control. GST-RBD– or GST-PBD–coupled beads were added to the remaining lysates for 35 minutes on a rotating platform at 4°C. Samples were washed three times, denatured, separated by electrophoresis, and transferred as described.

C3 Transferase Treatment. SUM-159 siRNA lines were cultured to 50% confluence in 6-well dishes and treated for 24 hours with human recombinant C3 Transferase (10 μg/mL; Cytoskeleton-CT03, Cytoskeleton, Denver, CO). Control untreated cells were cultured in parallel. Cells were harvested as described, washed three times in serum-free media, and invasion assays toward LPA (100 nmol/L; 1×10^5 cells) done as outlined above.

Three-Dimensional Matrigel Assays. Wells of a 24-well dish were precoated with 200 μL of undiluted phenol-red free Matrigel (10.2 mg/mL; BD Biosciences, San Jose, CA). Cells were harvested as described, washed three times with PBS, and diluted to a concentration of 1×10^4 per well in a volume of 200 μL. Cells were mixed with 100 μL of undiluted ice-cold Matrigel for a ratio 2:1, and laid over the bottom layer. After gelling, complete culture media was added and changed every 2 to 3 days. Morphology was assessed at day 11 by image capture at 10× magnification. For analysis of cell viability, the Matrigel was washed with PBS and digested by incubation with 500 μL of dispase (BD Biosciences) at 37°C for 2 hours. Cell aggregates were recovered by pipetting, diluted in 20 mL of PBS, and pelleted by centrifugation. A single cell suspension was obtained after trypsin-EDTA treatment for 5 minutes at 37°C. Cells were incubated with FITC-conjugated annexin-V (2.5 μg/mL; Biosource International, Camarillo, CA) for 20 minutes on ice, washed with 1× annexin-V buffer, and analyzed by flow cytometry.

RESULTS

Comparative Analysis of RhoA and RhoC Expression in Breast Cell Lines. Increased expression of *RhoC* at the mRNA level has been reported previously in a range of breast carcinoma cells (24);

[3] http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi.

however, the relative level of RhoA to RhoC expression has not been established. Because of the lack of a commercially available antibody specific for RhoC, we used a polyclonal Rho Ab that recognizes both RhoA and RhoC (see below) to examine expression of these isoforms. We screened breast carcinoma cell lines ranging from nonmotile to highly invasive and detected two bands that correspond to RhoA (~26 Mr) and RhoC (~27 Mr) in all lines (Fig. 1*A*). The relative expression of RhoA to RhoC varied for each cell line and neither the level of RhoA nor RhoC expression corresponded with increased invasiveness (Fig. 1*A*). We chose to pursue our studies using the SUM-159 cell line which was derived from a primary anaplastic breast tumor and is highly invasive both *in vitro* and *in vivo* (17). This line is distinct from inflammatory breast cancer and thereby represents an invasive cell line that may be less dependent on RhoC for its motility but more indicative of other highly invasive lines.

Generation of Cell Lines Expressing RhoA and RhoC siRNA. Despite the high level of sequence identity shared between RhoA and RhoC (25), we identified putative candidate sequences for the siRNA approach using Oligoengine software and confirmed their specificity by nucleotide BLAST searches. A negative control encoding the scrambled RhoA (Scr) siRNA sequence shared no homology to any human genes. Alignment of each siRNA sequence, beginning at nucleotide position 153 for RhoA, and position 193 for RhoC, revealed three nucleotide mismatches for each sequence (Fig. 1*B*). The sequences were synthesized as part of a 64 bp hairpin loop structure and cloned into the pSUPER.retro retroviral expression vector (Oligoengine). Viral stocks were generated and subsequently used to stably infect SUM-159 breast carcinoma cells.

Analysis of RhoA and RhoC expression in each siRNA line by semi-quantitative reverse transcription-PCR showed a specific reduction in mRNA levels for each gene relative to the Scr control (Fig. 1*C*). We observed no change in RhoB expression at the mRNA level (data not shown). Immunoblotting extracts from each siRNA line using a polyclonal Rho Ab revealed a complete reduction in expression of the appropriate isoform (Fig. 1*D*), confirming that this Ab recognizes both RhoA and RhoC (see Fig. 1*A*). Interestingly, we observed an increase in RhoC expression of 1.8-fold as a result of loss of RhoA expression and, conversely, a 2-fold increase in RhoA expression in the RhoC siRNA cells. Expression of Rac1 and Cdc42 remained unchanged in these lines. Importantly, loss of either RhoA or RhoC expression did not have a deleterious effect on the monolayer growth rate or survival of SUM-159 cells (data not shown).

RhoA and RhoC Have Distinct and Inverse Roles in Invasion. To investigate a role for RhoA and RhoC in invasion, we did Matrigel transwell assays using the siRNA cell lines. SUM-159 cells are highly invasive (17, 26). LPA, a component of serum and a known regulator of RhoA activation (15), stimulated robust invasion of the Scr control cells in a 2-hour assay (Fig. 2). This level of invasion was consistent with that observed for an empty vector pSUPER.retro control and the parental cell line (data not shown) indicating no functional change as a consequence of the Scr sequence. For all subsequent experiments, only the Scr control line was used. In comparison, a significant increase in invasion was observed for the RhoA siRNA cells. In contrast, the RhoC siRNA cells exhibited a significant decrease in invasion compared with the Scr control cells (Fig. 2). The reduction in invasion observed in the RhoC siRNA cells cannot be accounted for by cell death because no significant difference in the level of apoptosis, as measured by annexin-V staining, was observed (data not shown).

To extend these findings, MCF-7 cells were used to generate stable retroviral lines for RhoA siRNA and the Scr control. RhoA knockdown was robust (Fig. 3*A*) and resulted in a dramatic reduction in cell to cell contact and elongated morphology with large lamellae, indicative of motile cells (Fig. 3*B*). Consistent with this morphology, the

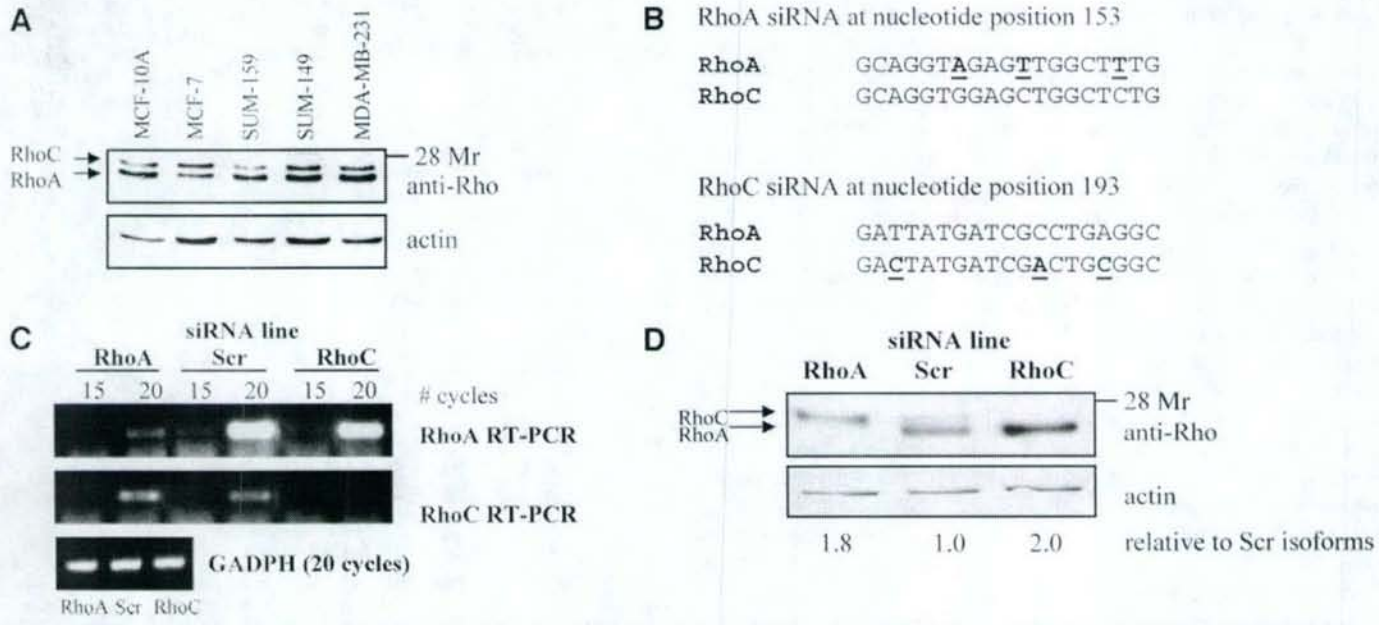

Fig. 1. Cell line comparisons and generation of RhoA- and RhoC-specific siRNA. *A*, immunoblotting of total protein lysates (35 μg) extracted from various breast cell lines. MCF-10A cells are nonmotile nontransformed mammary epithelial cells, MCF-7 cells are weakly invasive breast carcinoma cells, whereas SUM-159, SUM-149, and MDA-MB-231 cells are all highly invasive. The polyclonal Rho Ab detects both RhoA and RhoC isoforms as shown by the presence of two bands of approximately 26 and 27 kDa (indicated by *arrows*). *B*, alignment of each 19 bp Rho siRNA sequence. RhoA begins at nucleotide 153 and RhoC begins at nucleotide 193. Both sequences show three separate nucleotide mismatches as indicated in *bold type* and *underlined* and were determined by BLAST database analysis to be gene specific. *C*, semi-quantitative reverse transcription-PCR (15 and 20 cycles) of RNA (1-μg total) extracted from SUM-159 siRNA cells. In the RhoA siRNA cells, the mRNA levels at two independent cycles were significantly reduced relative to the Scr control and RhoC siRNA lines. Similarly, RhoC mRNA levels were significantly reduced in the RhoC siRNA line. The level of glyceraldehyde-3-phosphate dehydrogenase mRNA after 20 cycles was included as a control. *D*, immunoblotting of total protein lysates (35 μg) extracted from the siRNA cell lines. Both RhoA and RhoC bands are present in the Scr control lane (indicated by *arrows*). Accordingly, each siRNA lane shows a complete absence of the appropriate band. Densitometric comparisons of the individual Rho isoforms in the Scr control show a relative ratio of RhoA to RhoC expression of 2.6-fold. Comparison of expression of each isoform in the Scr control (set as 1) show a 1.8-fold increase in RhoA expression in the RhoC siRNA line and a 2-fold increase in RhoC expression in the RhoA siRNA line. Actin hybridization is included as a loading control.

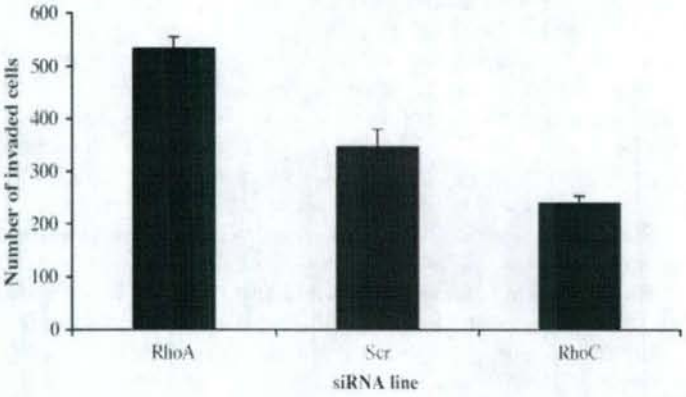

Fig. 2. RhoA expression is inhibitory to invasion, whereas RhoC expression is required for invasion. To determine the invasive capacity of the siRNA lines, cells (1×10^5) were loaded into the upper chamber of Matrigel-coated transwells and allowed to invade toward LPA (100 nmol/L) for 2 hours at 37°C. The average number of invaded cells in five independent fields was counted for each well and the mean of triplicate wells (±SD) is shown in this representative assay. RhoA siRNA cells invade significantly faster than the Scr control cells (54% in this assay; $^{**}P < 0.01$). In contrast, invasion was inhibited by 30% ($^{*}P < 0.05$) as a result of loss of RhoC expression. *P* values represent standard *t* test.

RhoA siRNA cells exhibited a substantial increase in migration (approximately 7-fold) toward NIH-3T3–conditioned media compared with the Scr control cells (Fig. 3*C*). These results confirm in two cell lines of vastly different invasive capacity that RhoA expression limits the migration and invasion capacity of breast carcinoma cells. Given the weakly invasive nature of these cells (17, 27), we did not investigate the loss of RhoC expression.

Rho GTPase Activity in the RhoA and RhoC siRNA Cells. An important issue that arises from the results presented above is the impact of RhoA and RhoC depletion on basal and LPA-stimulated Rho GTPase activation in the SUM-159 cells. These GTPase assays were done on cells adherent to laminin-1 because it is the major adhesive component of basement membranes, structures through which breast carcinoma cells invade. We obtained similar data using a collagen substratum. Cells adherent to laminin-1 for 60 minutes were stimulated with LPA for 3 minutes and the GTP-bound forms of RhoA and RhoC were detected in extracts from these cells by Rhotekin binding and immunoblot analysis of bound proteins. Activity was measured by densitometry and calculated as a ratio of the Rhotekin-bound to total protein for both isoforms. Values were normalized to the basal activity of the individual RhoA and RhoC isoforms in the Scr control cells. Analysis of the Scr control cells revealed that the basal activity of RhoA and RhoC was similar after attachment to laminin-1 for 60 minutes and that both isoforms were activated by LPA to similar levels (1.1-fold for RhoA and 1.2-fold for RhoC, Fig. 4*A* and *B*). In the RhoA siRNA cells, LPA treatment increased RhoC activity by 1.4-fold, slightly higher than the activity for the Scr control RhoC isoform (Fig. 4*B*). In the RhoC siRNA cells, activation of the RhoA isoform in response to LPA treatment increased by 2.6-fold, a substantial increase in comparison to the Scr control RhoA isoform (Fig. 4*B*). These data suggest that activation of each isoform is a codependent event because the loss of individual Rho isoform expression leads to differential changes in the ability to activate the remaining isoform after LPA stimulation.

Disruption of Rho Protein Signaling through C3 Treatment. To determine the contribution of the RhoA and RhoC proteins to the invasion observed after the Rho-specific knock-downs, we treated the siRNA lines with C3 transferase (10 μg/mL for 24 hours) and assessed their invasion toward LPA. The bacterial exotransferase from *C. botulinum* (C3 transferase) irreversibly ADP ribosylates RhoA, RhoB, and RhoC proteins, inhibiting downstream signaling interactions (15). C3 treatment of the Scr control cells slightly increased their invasive potential relative to untreated control cells (Fig. 5*A*), whereas the invasive potential of the RhoC siRNA cells was significantly enhanced after C3 treatment compared with the untreated cells (Fig. 5*B*). In contrast, invasion of the RhoA siRNA cells was significantly reduced by C3 treatment compared with untreated RhoA siRNA cells (Fig. 5*C*). However, the C3-treated RhoA siRNA cells were still more invasive than either the Scr control (by 2-fold) or RhoC siRNA C3-treated cells (data not shown). We infer from this approach that the increased expression of the opposing Rho isoform and subsequent activity in each Rho siRNA line contributes to the observed differences in invasion. Specifically, knock-down of RhoC by siRNA results in decreased invasion, and disruption of RhoC signaling by C3 treatment in the RhoA siRNA cells also results in a decrease in invasion. Similarly, disrupting RhoA signaling by C3 treatment in the

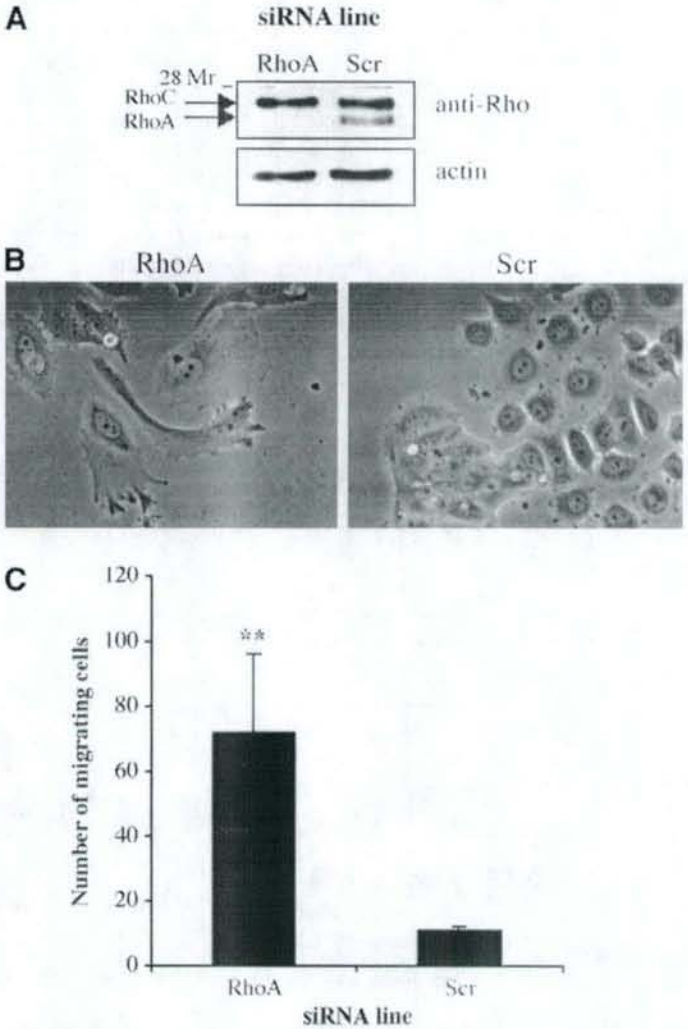

Fig. 3. Loss of RhoA expression in MCF-7 cells recapitulates SUM-159. MCF-7 cells were used to establish stable RhoA siRNA and Scr control cell lines. *A*, immunoblotting of total protein lysates (35 μg) by the polyclonal Rho Ab shows ablation of RhoA expression in the RhoA siRNA cells (individual isoforms are indicated by *arrows*). Actin hybridization is included as a loading control. *B*, phase-contrast images (10× magnification) of adherent cells growing on tissue culture plastic show the RhoA siRNA cells have a reduced requirement for cell to cell contact, are elongated, and have large lamellipodia. In comparison, the Scr control cells require cell to cell contact and exist in an epithelial colony network. *C*, analysis of the migratory potential of the RhoA siRNA cells compared with the Scr control by chemotaxis toward NIH-3T3 conditioned media for 24 hours. The RhoA siRNA cells showed a significant ($^{**}P < 0.01$) increase in migration of approximately 7-fold compared with the Scr control. The total number of migrating cells was counted for each well (in triplicate) and averaged for duplicate experiments (±SD). *P* values represent standard *t* test.

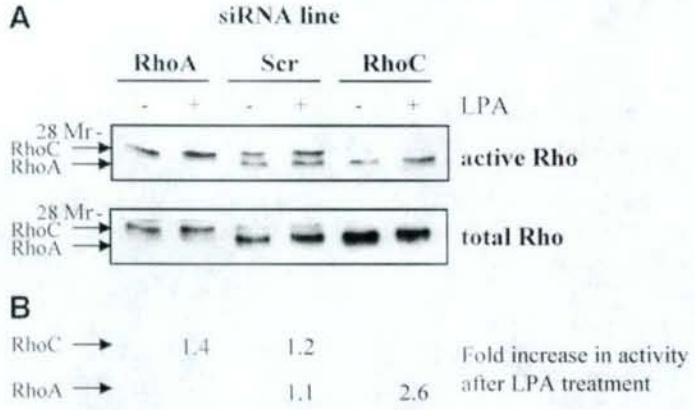

Fig. 4. Rho GTPase activities are distinct between siRNA lines. Rho activity assays were done on siRNA cells adherent to laminin-1 for 60 minutes followed by treatment with (+) or without (−) LPA for 3 minutes. Rho activity was measured by binding to the GST-rhotekin fusion protein and hybridization with the polyclonal Rho Ab. A proportion of the total cell lysate was used to control for loading accuracy. A representative assay is shown. *A*, the Rho activity assay shows that both RhoA and RhoC are active after adhesion to the substratum and that their activity is increased in response to LPA treatment. The RhoA (~26 kDa) and RhoC (~27 kD) isoforms are indicated by *arrows*. *B*, Rho isoform activity was quantitated by densitometry and is presented as the fold increase in activity after LPA treatment. In the RhoA siRNA cells, the activity of the RhoC isoform after LPA treatment showed a slight increase (1.4-fold) in comparison to the Scr control RhoC isoform (1.2-fold). Activation of RhoA in response to LPA treatment of the RhoC siRNA cells was significantly higher (2.6-fold) than the Scr control RhoA isoform (1.1-fold).

RhoC siRNA cells results in an increase in invasion, as seen in the RhoA siRNA cells. Thus, decreased invasion in the RhoC siRNA cells can be attributed to RhoA activity, whereas increased invasion in the RhoA siRNA cells can be attributed in part to RhoC activity. Given that the C3-treated RhoA siRNA cells continue to invade significantly faster than the Scr control C3 treated cells, other factors are likely influencing invasion in this line in addition to the Rho proteins.

Distinct Morphology and Adhesion of the RhoA and RhoC siRNA Cells. To gain insight into the distinct functions of RhoA and RhoC that could account for their opposing influences on invasion, we examined the morphology of the siRNA cells adherent to laminin-1 after stimulation with LPA. Scr control cells exhibited active membrane ruffling after attachment to laminin-1 for 60 minutes (data not shown), and increased ruffling and the formation of small lamellipodia were evident in response to LPA stimulation for 3 minutes (Fig. 6*A*). RhoA siRNA cells also showed active ruffling during attachment but formed extended, fine, veil-like lamellipodia in response to LPA treatment (Fig. 6*A*). Quantitation of the total lamellipodial area after LPA stimulation showed a significant increase in the RhoA siRNA cells (Fig. 6*B*). In contrast, the majority of the RhoC siRNA cells were less well spread compared with the Scr control and showed extensive membrane ruffling with limited lamellipodia formation and evidence for collapsed lamellae (Fig. 6*A*). No significant difference in total lamellipodial area compared with Scr control cells was determined (Fig. 6*C*). Intense phalloidin staining was seen at the edges of the RhoC siRNA cells, which is also indicative of extensive membrane ruffling (Fig. 6*A*). Interestingly, phalloidin staining showed that loss of either Rho protein did not impair the formation of actin stress fibers in response to LPA treatment (Fig. 6*A*). This observation suggests that all Rho proteins are equally capable of stress fiber formation or that compensation for this function exists between the Rho proteins. Taken together, the results suggest that loss of RhoA expression increases the size of lamellipodia that form in response to LPA. Increased membrane ruffling in the RhoC siRNA cells may be indicative of lamellipodia that are unable to extend, possibly explaining their limited motility. The observation that adhesion of the RhoA siRNA cells to laminin-1 after 60 minutes was significantly reduced compared with the Scr control and RhoC siRNA cells (Fig. 6*C*) is indicative of more motile cells and also suggests a functional role for RhoA in adhesion.

Rac1 GTPase Activity Is Differentially Regulated in the RhoA and RhoC siRNA Cells. Given that inhibition of Rho proteins by C3 treatment did not reduce the invasive capacity of the RhoA siRNA cells to the level of the Scr control, the interplay of other factors contributing to invasion is likely. The increased lamellipodial area observed in the RhoA siRNA cells in response to LPA stimulation is suggestive of increased Rac1 activity. Rac1 has been shown previously to be responsive to LPA (28). To establish whether the reported reciprocity in RhoA and Rac1 activity (29) might correlate with the increased invasion observed in the absence of RhoA expression, Rac1 activity assays were done under the same conditions as the Rho activity assays, and activity was measured as described previously (23). No change in total Rac1 expression was observed between the cell lines. Treatment with LPA for 3 minutes increased Rac1 activity in the Scr control cells by approximately 1.3-fold (Fig. 7). In contrast, Rac1 activation in the RhoA siRNA cells increased by a striking 2.5-fold after LPA treatment, whereas activity in the RhoC siRNA cells resulted in a 1.4-fold increase (Fig. 7). These data suggest there is cross-talk between RhoA and Rac1 and that RhoC does not impact Rac1 activity to the same extent.

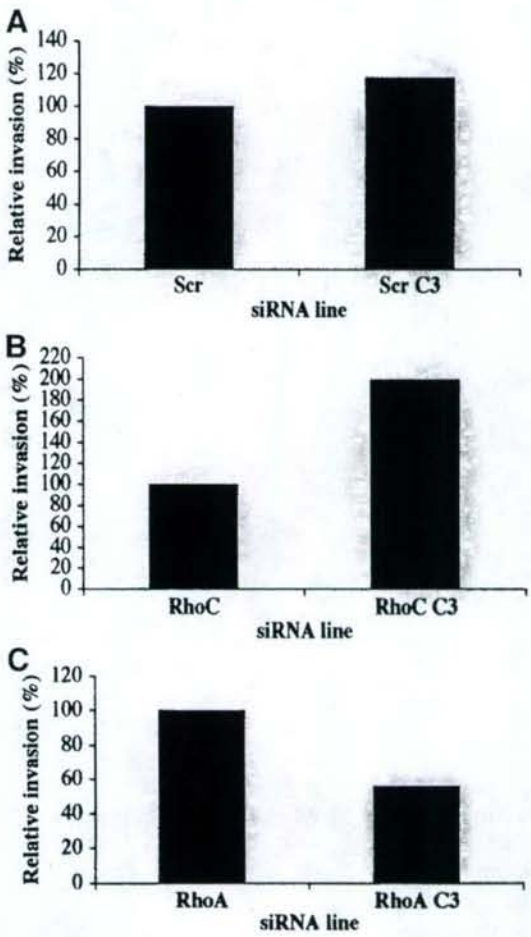

Fig. 5. C3 transferase treatment of siRNA lines alters invasive potential. Adherent cells at 50% confluence were treated with C3 transferase (10 μg/mL in standard growth media) for 24 hours, harvested, and allowed to invade Matrigel-coated transwells toward LPA for 2 hours. The average number of invaded cells was determined from five independent fields for triplicate wells and is represented as relative invasion, setting the level of invasion for the untreated cells of each siRNA line to 100%. A representative assay is shown. *A*, the C3-treated Scr control cells do not vary significantly from untreated cells. *B*, in contrast, C3 treatment significantly enhanced invasion of the RhoC siRNA cells. *C*, C3 treatment reduced invasion of the RhoA siRNA cells.

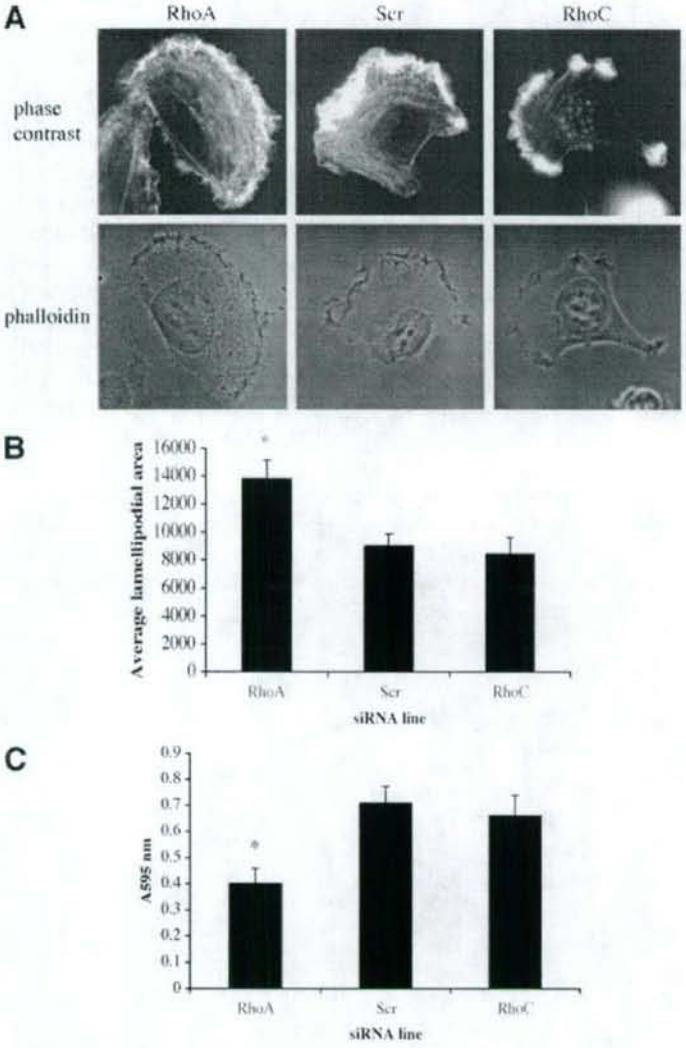

Fig. 6. Morphological effects of Rho siRNA. The morphology of the siRNA cells was determined after adhesion to laminin-1 for 60 minutes followed by treatment with LPA for 3 minutes. *A*, phase-contrast images of the cells and FITC-conjugated phalloidin staining. Scr control cells showed increased membrane ruffling leading to the formation of small lamellipodia whereas RhoA siRNA cells exhibited veil-like extended lamellipodia in response to LPA treatment. The absence of RhoC expression resulted in extensive ruffling around the cell body and limited lamellae formation. LPA treatment induced formation of a stress fiber network, although no significant difference was observed among the three lines. All images were taken at 60× magnification. *B*, quantitation of the lamellipodial area of >20 individual cells in response to LPA treatment shows a significant increase in area for the RhoA siRNA cells ($*P < 0.05$) compared with the Scr control cells. Areas were quantitated by IPLab Spectrum software. The average lamellipodial area is presented (±SD). *C*, adhesion of the siRNA cells to laminin-1 for 60 minutes was determined by crystal violet staining of cells after washing. The crystal violet absorbed by the cells was solublized, and the absorbance was measured at A595 nm. The average of triplicate determinations is shown (±SD). RhoA siRNA cells were significantly less adhesive ($*P < 0.05$) compared with the Scr control and RhoC siRNA cells. *P* values represent standard *t* test.

Contribution of Rho Proteins to Three-Dimensional Morphology. To investigate Rho protein function in a more physiologically relevant setting, the siRNA cells were embedded in Matrigel. Matrigel mimics the mammary gland environment, being rich in basement membrane components such as laminin, collagen IV, heparan sulfate proteoglycans, and growth factors. Cells were embedded at low density and supplemented with standard growth medium for 11 days. The RhoA siRNA cells formed dense aggregates from which highly invasive cells emanated, forming a stellate morphology that almost completely filled the well by the termination of the experiment (Fig. 8). In comparison, the Scr control cells formed a combination of medium-sized cellular aggregates with disorganized edges and small stellate outgrowths. RhoC siRNA cells, however, formed very small aggregates with limited invasive projections (Fig. 8). Cell death was excluded as the reason for reduced aggregate formation in the RhoC siRNA cells because no significant difference in the proportion of annexin-V–positive cells was seen (Fig. 8). These data substantiate the invasion assay data (Fig. 2), from which it would be predicted that the RhoC siRNA cells would be significantly impeded in their capacity to penetrate the Matrigel and conversely, the RhoA siRNA cells would be unrestricted.

DISCUSSION

We have used breast carcinoma cells and a RNA interference (RNAi) approach to dissect the functions of RhoA and RhoC, two Rho isoforms that share high sequence identity. Ablation of the expression of these Rho isoforms individually in SUM-159 cells, which are invasive and characteristic of late-stage tumors, enabled us to deduce that RhoA impedes and RhoC stimulates invasion. In addition, diminution of RhoA expression in the relatively nonmotile cells, MCF-7, induced their ability to migrate quite markedly. Together, these findings indicate that RhoA impedes the migration and invasion of breast carcinoma cells, a function distinct from RhoC. However, given that RhoA expression is maintained in invasive breast carcinomas (4), our findings argue that RhoA may contribute in other ways to breast cancer progression. For example, RhoA has been implicated in the epithelial-mesenchymal transition of carcinoma (30). In addition to demonstrating distinct functions for RhoA and RhoC, our studies unexpectedly revealed a compensatory relationship between RhoA and RhoC at the level of both their expression and activation.

Our findings should be considered within the context of the existing literature on Rho isoform expression in breast cancer. These studies have focused largely on the assessment of RhoA and RhoC expression in both cell lines and tumor specimens. A prevailing notion is that RhoC expression correlates with aggressive disease as evidenced by high levels of RhoC expression in inflammatory breast cancers, which are exceptionally aggressive tumors (31), and increased motility of normal mammary epithelial cells over-expressing RhoC (8). Clearly, our RNAi data implicating a proinvasive role for RhoC substantiate this notion. We note, however, that both RhoA and RhoC are expressed in all of the breast carcinoma cell lines we have analyzed and that neither of their expression patterns correlates strongly with invasive potential (Fig. 1*A*). Moreover, the ratio of RhoA to RhoC ex-

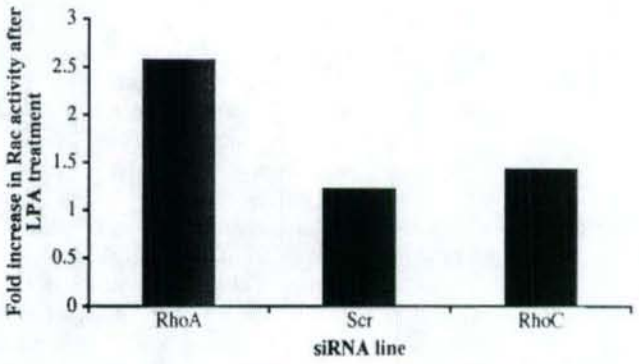

Fig. 7. Rac1 GTPase activity is distinct between siRNA lines. Rac activity assays were done on siRNA cells adherent to laminin-1 for 60 minutes followed by treatment with (+) or without (−) LPA for 3 minutes. Activity was measured by binding to the GST-PBD fusion protein followed by detection with the monoclonal Rac1 Ab. An aliquot of the total cell lysate was used as a control for loading. Densitometric quantitation was done, and the activity was calculated as a proportion of the total protein, setting the Scr control as 1. The fold increase in activity after LPA treatment is indicated for each cell line and shows that induction of Rac1 activity is significantly higher in the RhoA siRNA cells.

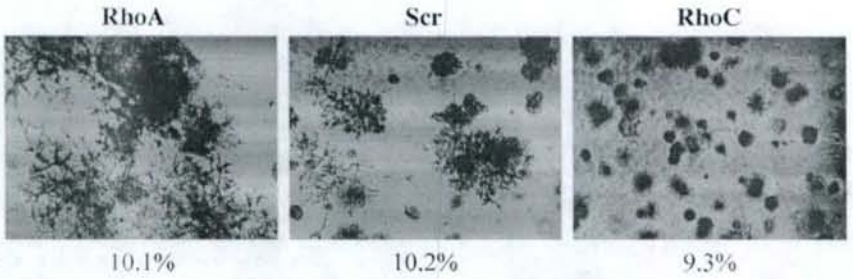

Fig. 8. Morphology in three-dimensional Matrigel reflects invasive capacity. Cells were embedded at low density in Matrigel and cultured in the presence of growth media for 11 days. Phase-contrast images show the formation of dense cell aggregates and highly penetrating stellate cells in the RhoA siRNA whereas the Scr control cells aggregate and form protrusions but to a much lesser extent. In contrast, the RhoC siRNA cells formed very small aggregates with limited protrusions. Cell viability remained unchanged between the cell lines as indicated by the low percentage of cells that stained annexin-V positive. All images were taken at 10× magnification.

pression varies within these lines. This factor may be a critical determinant of functionality, particularly given the interplay observed between these proteins in the siRNA lines. This observation suggests that the regulation of RhoC function may be more complex than relative expression and that the RNAi approach we have developed will be useful to assess RhoC function in different types of breast carcinoma cells. Furthermore, studies that have concluded that RhoA expression increases in breast tumors are based either on the analysis of mRNA expression that may not necessarily reflect protein expression, or those studies have used Abs that may recognize multiple isoforms of Rho proteins. Despite these caveats, it is likely that RhoA contributes to aspects of breast tumorigenesis and progression other than invasion itself. Again, the RNAi approach will enable the elucidation of such contributions.

The compensatory relationship between RhoA and RhoC expression that we uncovered is unexpected and intriguing. The observation that this compensation occurs at the protein but not the mRNA level suggests a post-transcriptional mechanism that facilitates RhoC expression in the absence of RhoA and *vice versa*. This compensation is difficult to reconcile in light of our finding that these two isoforms have distinct functions. Indeed, the findings that loss of RhoC expression results in increased RhoA expression and activation and that this increase in RhoA activation impedes the invasion of the RhoC RNAi cells, as evidenced by the C3 data, indicate that the analysis of Rho isoform function is less straightforward than anticipated. This assessment has important implications for the generation and analysis of Rho knockout mice.

The regulation of RhoA and RhoC activation and the mechanisms by which these GTPases influence cell functions differently are two key issues that need to be addressed. Although RhoC has been implicated in the progression of many invasive carcinomas, its functional activation had not been reported. We show here that it is activated in response to LPA treatment, which also activates RhoA. Given their high sequence similarity, it is not surprising that the Rho proteins share similar modes of regulation. It will be interesting to determine whether RhoA and RhoC share the same exchange factors and, if so, whether the loss of expression of one isoform alters the balance of free guanine nucleotide exchange factors in favor of the remaining isoform. In this context, it is also notable that the exchange factor XPLN, detected in limited tissues, is specific for RhoA and RhoB but not for RhoC (32). What has not been addressed rigorously is the likely possibility that endogenous RhoA and RhoC differ in their subcellular localization, especially after activation. The RNAi approach, combined with fluorescent resonance emission technology (33), could be used to spatially localize the activated form of one isoform in the absence of the other.

Our RNAi data suggest distinct functions for RhoA and RhoC. In support of our data, recent studies using an overexpression model indicate that RhoA and RhoC differentially interact with the downstream effector Rho kinase (ROCK): RhoC exhibits a higher affinity for ROCK than does RhoA (34). ROCK, however, is one of the many downstream effector molecules that are known to interact with RhoA, and other examples of differential interactions are likely. Our data also suggest compensation functions exist within this family because no significant difference in stress fiber formation was observed after LPA treatment in either siRNA line, suggesting that both RhoA and RhoC have the capacity to regulate stress fibers. Indeed, in porcine endothelial cells active RhoA and RhoC equally induce stress fiber formation (35). Nonetheless, the critical issue from our perspective is the opposing effects of these two GTPases on breast carcinoma invasion. The fact that the RhoA and RhoC sequences differ significantly only in their COOH-termini (25) argues that the opposing effects of these two isoforms on invasion resides in these COOH sequences. The siRNA cells we have generated should be quite useful for assessing this hypothesis. For example, expression of COOH-terminal mutants of RhoC in the RhoC siRNA cells should facilitate the identification of specific RhoC amino acids that contribute to an invasive phenotype.

Although cross-talk between Rho and Rac activity has been described (23, 29, 36), no studies have addressed the involvement of individual Rho isoforms in this cross-talk specifically. In the absence of RhoA expression, we observed a significant increase in Rac1 activity after LPA treatment and increased lamellipodia formation consistent with a Rac1 induced phenotype (37). In preliminary studies, p21 activated kinase, a downstream Rac1 effector molecule was similarly activated by the loss of RhoA but not RhoC (data not shown). This finding is in agreement with reports on reciprocity between RhoA and Rac1 activities, with high RhoA activity being inhibitory to Rac1 activity and low RhoA activity resulting in high Rac1 activity and cell motility (29). C3 treatment of the RhoA siRNA cells partially reduced the invasive capacity of the RhoA siRNA cells, suggesting that the increased Rac1 activity may account for a proportion of the increased invasion. In contrast, disrupting RhoC expression did not impact Rac1 activity significantly, despite the high RhoA activity in these cells. Taken together, these results suggest that RhoA but not RhoC can influence Rac1 activation and intimates that these proteins can impact independent pathways. The mechanism involved awaits further investigation.

In summary, we have shown the successful application of RNAi to assess the functions of RhoA and RhoC in invasive breast carcinoma cells. The results obtained provide significant insight into their functions and suggest it is no longer sufficient to attribute Rho-associated functions to RhoA alone. This approach is directly applicable to a broad range of studies on Rho protein biology and should prove useful in dissecting the activation and downstream signaling pathways influenced by these GTPases. In addition, our results suggest that the

differential targeting of these isoforms may be feasible for therapeutic purposes, especially in light of the fact that RNAi is currently being developed for clinical therapies (reviewed in ref. 38). Moreover, the use of stable retroviral incorporation of the siRNA provides a platform to extend our studies to orthotopic *in vivo* models to establish the functional role of these proteins in breast cancer progression.

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